Mycologia, 98(5), 2006, pp. 737–745.

Metarhizium frigidum sp. nov.: a cryptic species of *M. anisopliae* and a member of the *M. flavoviride* complex

Joseph F. Bischoff[1]

National Center for Biotechnology Information, National Institutes of Health, Bethesda, Maryland 20894, and Insect Biocontrol Laboratory, USDA, ARS, BARC-West, Beltsville, Maryland 20705-2350

Stephen A. Rehner

Insect Biocontrol Laboratory, USDA, ARS, BARC-West, Beltsville, Maryland 20705-2350

Richard A. Humber

USDA/ARS Plant, Soil and Nutritional Laboratory, Ithaca, New York 14853

Abstract: The anamorph genus *Metarhizium* is composed of arthropod pathogens, several with broad geographic and host ranges. Members of the genus, including ''*M. anisopliae* var. *frigidum*'' nomen nudum and *Metarhizium flavoviride*, have been used as biological insecticides. In a recent revision of the genus the variety ''*M. anisopliae* var. *frigidum*'' was suggested to be a synonym of *M. flavoviride* based largely on ITS sequence phylogenetic analysis. In this study we conducted morphological evaluations and multigene phylogenetic analyses with EF-1α, RPB1 and RPB2 for strains of *M. flavoviride* and ''*M. anisopliae* var. *frigidum*.'' Included in these evaluations were the ex-type of *M. flavoviride* var. *flavoviride* and what likely would be considered the ''ex-type' of the invalidly published taxon ''*M. anisopliae* var. *frigidum*''. Based on morphological and molecular evidence we conclude that ''*M. anisopliae* var. *frigidum*'' is distinct from *M. flavoviride* and the taxon *M. frigidum* sp. nov. is described.

Key words: biocontrol, Clavicipitaceae, *Cordyceps*, cryptic species, entomopathogen

INTRODUCTION

The type species of *Metarhizium* Sorokin, *M. anisopliae* (Metschn.) Sorokin, was described from Russia as a pathogen of wheat cockchafer, *Anisolia austriaca* (Metchnikoff 1879). Members of this cosmopolitan genus are entomopathogens of a broad range of arthropod orders. The pathogen enters the host body by forming an appressorium and using a penetration peg to gain access, after which the internal tissues of the host are consumed (Sajap and Kaur 1990). After this mycelium and conidiophores develop on the outside of the corpse where green-pigmented conidia are produced en masse. It was from this latter feature that members of the genus were given the common name ''green muscardine fungus.''

In her 1976 revision of the genus Tulloch recognized only two species, *M. anisopliae* and *M. flavoviride* W. Gams & Rozsypal, along with a single variety *M. anisopliae* var. *majus* (J.R. Johnst.) M.C. Tulloch (as var. major). Since that time additional species and varieties have been identified and links have been made to the teleomorph genus *Cordyceps* (Fr.) Link of the hypocrealean family Clavicipitaceae (Liang et al 1991, Liu et al 2001). In the most recent revision of the genus Driver et al (2000) recognized three species and six varieties. However they were unable to include four previously described species of *Metarhizium* in their study because ''…none is [sic] known to be deposited in culture collections'' (Driver et al 2000 p 136). These unrepresented taxa are *M. pingshaense* X.T. Chen & H.L. Guo, *M. cylindrosporae* X.T. Chen & H.L. Guo (=*Nomuraea cylindrosporae* [X.T. Chen & H.L. Guo] Tzean, L.S. Hsieh, J.L. Chen & W.J. Wu), *M. guizhouense* X.T. Chen & H.L. Guo, and *M. taii* Z.Q. Liang & A.Y. Liu (anamorph of *Cordyceps taii* Z.Q. Liang & A.Y. Liu). Driver et al (2000) did not comment on the positions and validity of these taxa. In addition they suggested that ''*M. anisopliae* var. *frigidum*'' was actually a synonym of *M. flavoviride.* Their analyses were based on internal transcribed spacer (ITS) regions 1, 5.8S, 2 and the D3 region of 28S (LSU) of the nuclear ribosomal DNA (rDNA).

Yip et al (1992) investigated conidial measurements, growth response to temperature regimes, and pathogenicity on scarab larvae of 204 isolates identified as *Metarhizium anisopliae* var. *anisopliae.* They found that all of these isolates shared similar conidial morphology but some showed the ability to germinate at colder temperatures (i.e. 5 C). Using these data and adding carbohydrate use patterns Rath et al (1995) also identified particular strains that were ''cold-active'' and referred to them as ''*Metarhizium anisopliae* var. *frigidum*''.

When including a broader sample of *Metarhizium* taxa, Driver et al (2000) determined that ''*Metarhi-*

Accepted for publication 10 Aug 2006.

[1] Corresponding author. National Center for Biotechnology Information, National Institutes of Health, Bethesda, Maryland 20894. E-mail: bischoff@ncbi.nlm.nih.gov

zium anisopliae var. *frigidum*'' was more closely related to the *Metarhizium flavoviride* clade than *M. anisopliae*. The D3 region of the LSU and ITS rDNA regions used in their phylogenetic study did not support a clear distinction between ''*Metarhizium anisopliae* var. *frigidum*'' and *Metarhizium flavoviride*, so they were recognized as synonyms. However Driver et al did not address the morphological disparities between the conidial measurements of ''*M. anisopliae* var. *frigidum*'' (Yip et al 1992, Rath et al 1995) and *M. flavoviride*, with its larger conidia (Gams and Rozsypal 1973; see TABLE I).

Driver et al (2000) commented on the lack of resolution provided by the D3 LSU and ITS rDNA regions. Because many of the lineages and the infrageneric relationships between them were not clearly resolved they described some terminal lineages as new varieties rather than new species. In this study we used higher resolution molecular markers and morphological evidence to determine whether ''*Metarhizium anisopliae* var. *frigidum*'' is a synonym of *M. flavoviride*, as suggested by Driver et al (2000), or a distinct lineage worthy of taxonomic recognition. We analyzed the full-length DNA sequence of the translation elongation factor (EF-1α) protein coding gene and partial sequences from two subunits of RNA polymerase II (RPB1 and RPB2). Due to the generosity of Richard Milner of the Commonwealth Scientific and Industrial Research Organization (CSIRO, Canberra, Australia), we have studied many of the isolates used in the Driver et al (2000) revision as well as ex-type cultures of *Metarhizium flavoviride* and ''*M. anisopliae* var. *frigidum*''.

MATERIALS AND METHODS

Fungal isolates.—A total of 33 isolates (see TABLE I) were obtained from the Agricultural Research Collection of Entomopathogenic Fungal Cultures (ARSEF, Ithaca, NY, USA) and the CSIRO, Canberra, Australia, for this study. Included among the isolates were the ex-types of *M. anisopliae* (ARSEF 7487 = FI 1029; neotype [Tulloch 1976]), ''*M. anisopliae* var. *frigidum*'' (ARSEF 4124 = DAT F-001), *M. flavoviride* var. *flavoviride* (ARSEF 2133) and *M. flavoviride* var. *minus* Rombach, Humber & Roberts (ARSEF 2037). The ex-type of *M. flavoviride* var. *pemphigi* Driver & Milner was unavailable for this study but ARSEF 7491 (=FI 1101), an authentic strain included in Driver et al (2000) was used.

Morphological evaluations.—Isolates were grown on quarter strength SDAY (SDAY/4) media (SDAY: 10 g bactopeptone, 40 g dextrose, 10 g yeast extract, 2% agar; Goettel and Inglis 1997) 5–14 d in the dark. Morphological observations and photographs of the cultured isolates were made with a Nikon Eclipse E600 compound microscope and ACT-1 version 2.12 image capturing software (Nikon Corp.). Minimum and maximum values in TABLE I are from a minimum of 20 measurements.

DNA extraction.—Isolates were grown in SDY/4 broth 5–7 d on an orbital shaker set at 125 rpm and 25 C. The tissue was removed from the broth, rinsed twice with sterile water, filter dried, frozen at −80 C and lyophilized. Approximately 50 mg of lyophilized mycelium was ground into powder with the FastPrep tissue homogenizer (MP Biomedicals, Irvine California). The pulverized tissue was lysed with 900 μL of lysis buffer (2 M NaCl, 0.4% w/v deoxycholic acid, 1.0% w/v polyoxyethylene ether) and incubated 10 min at 55 C. Cellular byproducts were extracted with 750 μL of chloroform: isoamyl alcohol (24:1) and centrifuged to separate the aqueous and particulate phases. The 700 μL of the cleared solution containing DNA was removed, placed in a clean tube and mixed with an equal portions of 6 M guanidinium isothiocyanate. DNA was bound to 40 μL of equal volumes of diatomaceous earth and flint glass powder. The bound DNA was resuspended twice in 75% ethanol, dried and eluted in sterile distilled water incubated 5 min at 55 C.

PCR amplification and nucleotide sequencing.—Partial sequences of three nuclear protein coding genes were amplified and sequenced for this study. They include the 5′ intron-rich region of the translation elongation factor 1-alpha (EF-1α intron region), a large exon region of the translation elongation factor 1-alpha (EF-1α exon), polymerase II largest subunit (RPB1) and two regions of the RNA polymerase second largest subunit (RPB2a and RPB2b). The 5′ EF-1α intron region was amplified and sequenced for all isolates while the other regions were amplified and sequenced for a subset (i.e. 15 isolates). The primers used for amplification and sequencing were 5′ EF-1α intron region: EF1T (5′-ATGGGTAAGGARGACAAGAC) and EF2T (5′-GGAAGTACCAGTGATCATGTT) (Rehner and Buckley 2005); EF-1α exon: 983F (5′-GCYCCYGGHCAYCGTGAYTTYAT), and 2218R (5′-ATGACACCRACRGCRACRGTYTG) (Rehner and Buckley 2005); RPB1: RPB1Af (5′-GARTGYCCDGGDCAYTTYGG) and RPB1C (5′-CCNGCDATNTCRTTRTCCATRTA) (Stiller and Hall 1997); RPB2a: fRPB2-5F (5′-GAYGAYMGWGATCAYTTYGG) and RPB2-7cR (5′-CCCATRGCTTGYTTRCCCAT) (Liu et al 1999); RPB2b: fRPB2-7cf (5′-ATGGGYAARCAAGCYATGGG) (Lui et al 1999) and RPB2-3053R (5′-TGRATYTTRTCRTCSACCATRTG) (Reeb et al 2004). The amplification primers and three additional primers for the EF-1α exon, 1567R (5′-ACHGTRCCRATACCACCRAT), 1577F (5′-CARGAYGTBTACAAGATYGGTGG), and 2212R (5′-CCRAACRGCRACRGTYYGTCTCAT) (Rehner and Buckley 2005), were used for sequencing. Procedures for amplification and sequencing were the same as used by (Rehner and Buckley 2005).

Sequence alignment and phylogenetic analyses.—Sequences were assembled and edited with Sequencher 4.1 (Gene Codes Corp., Ann Arbor, Michigan). Alignments were made with Clustal X (Thompson et al 1997) using the default settings. Adjustments to the alignment and the elimination of ambiguous regions were necessary only for the EF-1α intron region.

TABLE I. Strain code, location of collection, isolation source, GenBank numbers, and morphological measurements of species used in this study

				GenBank accession numbers					
				EF-1 alpha					
Species	Strain code	Country	Host	Intron region	Exon region	RPB1	RPB2	Conidia (µm)	Phialides (µm)
Metarhizium anisopliae	ARSEF 727	Brazil	Orthoptera	NA	DQ463994	DQ468353	DQ468368		
	ARSEF 3210	India	Coleoptera	NA	DQ463995	DQ468354	DQ468369		
	ARSEF 7487	Eritrea, Eastern Africa	Orthoptera	NA	DQ463996	DQ468355	DQ468370	5.0–7.0 × 2.0–3.5	8.0–11.5 × 2.0–3.0
M. flavoviride var. *flavoviride*	ARSEF 1184	France	Coleoptera	DQ463984	DQ463997	DQ468356	DQ468371		
	ARSEF 2024	France	Coleoptera	DQ463965	DQ464001	DQ468360	DQ468375	9.5–11.5 × 3.5–5.0	7.0–15.0 × 3.0–4.5
	ARSEF 2025	Germany	Agricultural Soil	DQ463992	DQ464000	DQ468359	DQ468374	8.0–11.0 × 3.5–4.5	11.5–17.0 × 2.5–4.5
	ARSEF 2026	Netherlands	soil	DQ463967	NA	NA	NA	9.0–11.5 × 4.5–6.0	10.5–16.0 × 2.5–4.0
	ARSEF 2133	Czech Rep.	Coleoptera	DQ463988	DQ463999	DQ468358	DQ468373		
	ARSEF 4272	Australia	soil	DQ463970	DQ463998	DQ468357	DQ468372	8.5–12.0 × 4.0–5.5	10.0–19.0 × 3.5–5.5
	ARSEF 4719	Australia	Coleoptera	DQ463980	NA	NA	NA	8.0–10.5 × 3.0–4.5	7.0–13 × 2.0–3.5
	ARSEF 4729	Australia	soil	DQ463986	NA	NA	NA		
	ARSEF 4730	Australia	soil	DQ463990	NA	NA	NA	8.0–12.0 × 3.5–5.0	10.5–17.0 × 2.5–3.5
M. flavoviride var. *pemphigi*	ARSEF 7491	United Kingdom	Homoptera	DQ463964	DQ464005	DQ468364	DQ468379		
	ARSEF 6569	United Kingdom	Homoptera	NA	DQ464004	DQ468363	DQ468378		
M. flavoviride var. *minus*	**ARSEF 2037**	Philippines	Homoptera	DQ463979	DQ464007	DQ468366	DQ468381		
	ARSEF 1764	Solomon Islands	Homoptera	NA	DQ464006	DQ468365	DQ468380		
M. frigidum	**ARSEF 4124**	Australia	Coleoptera	DQ463978	DQ464002	DQ468361	DQ468376	4.5–7.5 × 2.5–3.5	
	ARSEF 4219	Australia	soil	DQ463982	NA	NA	NA	5.0–7.5 × 2.5–3.5	6.0–11.0 × 2.0–3.5
	ARSEF 4277	Australia	soil	DQ463966	NA	NA	NA	5.5–7.5 × 2.0–3.5	7.0–13.5 × 2.0–3.5
	ARSEF 4294	Australia	soil	DQ463975	NA	NA	NA	5.5–7.5 × 2.5–3.5	4.0–11.0 × 2.0–3.5
	ARSEF 4561	Australia	soil	DQ463969	NA	DQ468367	DQ468382	4.5–7.5 (–9) × 3.0–4.0	6.5–12.5 × 2.5–4.0
	ARSEF 4765	Australia	soil	DQ463993	NA	NA	NA	6.0–8.0 × 2.5–3.5	8.0–11.5 × 2.5–3.5
	FI 733	Australia	Termite mound	DQ463971	NA	NA	NA	4.5–6.0 × 2.5–3.5	3.5–9.0 × 2.0–3.0
	FI 737	Australia	Termite mound	DQ463968	NA	NA	NA		
	FI 746	Australia	Termite mound	DQ463991	NA	NA	NA		
	FI 747	Australia	Termite mound	DQ463977	NA	NA	NA		
	FI 748	Australia	Termite mound	DQ463981	NA	NA	NA		
	FI 758	Australia	Termite mound	DQ463976	NA	NA	NA		
	FI 761	Australia	Termite mound	DQ463973	NA	NA	NA		
	FI 764	Australia	Termite mound	DQ463985	NA	NA	NA		
	FI 776	Australia	Termite mound	DQ463987	NA	NA	NA		
	FI 777	Australia	Termite mound	DQ463989	DQ464003	DQ468362	DQ468377		
	FI 783	Australia	Termite mound	DQ463983	NA	NA	NA		
	FI 785	Australia	Termite mound	DQ463974	NA	NA	NA		
	FI 793	Australia	Termite mound	DQ463972	NA	NA	NA		

Type strains in **Bold**. NA = region not sequenced for this study.

Maximum parsimony (MP) and Bayesian inference (BI) methods were used to develop phylogenetic hypotheses. MP-based analyses were done with PAUP* v.4.0b10 (Swofford 2002) using heuristic searches of 500 random-addition replicates with TBR branch swapping and equal character weighting. Heuristic MP bootstrap analyses (Felsenstein 1985) with TBR branch swapping included 1000 pseudoreplicates and 10 random addition replicates were done to identify bootstrap support values (BP).

Bayesian analyses were performed with MrBayes v.3.1 (Huelsenbeck 2000, Ronquist and Huelsenbeck 2003) to determine posterior probabilities (PP). MrBayes was run with 4 mcmc chains (3 cold, 1 heated) for 2 000 000 generations, sampling every 100 generations (including the first generation) for a total of 20 001 trees. The first 20% of the resulting trees were discarded to let the log-likelihood scores reach stability (i.e. ''burn in''). MrBayes was run twice in simultaneous, independent analyses starting from different random trees (default setting), providing a total of 36 000 trees. The trees were imported into PAUP and a 50% consensus tree was computed with the support values representing the PP values. Clades with 70% or greater BP and 95% PP or greater support were considered significantly supported by the data (Mason-Gamer and Kellog 1996, Reeb et al 2004).

EF-1α intron region, EF-1α exon, RPB1, and RPB2 were first analyzed individually. Examinations for topological incongruence were made between the EF-1α, RPB1 and RPB2 regions by a reciprocal 70% BP and a 95% PP (Reeb et al 2004) to determine if the datasets could be combined. Due to the rapidly evolving nature of the 5′ EF-1α intron region the most distal lineage from the *Metarhizium flavoviride* clade (i.e. *M. anisopliae*, Driver et al 2000) was not included in the combined gene analysis.

RESULTS

Morphological observations.—The pigmentation of conidiating cultures of ''*Metarhizium anisopliae* var. *frigidum*'' were distinctly darker green (28E7; Kornerup and Wanscher 1967; see FIG. 9) than that from isolates of *M. flavoviride* (29A3; Kornerup and Wanscher 1967; see FIG. 10) and more closely resembles that of *M. anisopliae* (FIG. 11). In addition the conidia and phialide measurements of ''*M. anisopliae* var. *frigidum*'' more closely resemble those from *M. anisopliae* (see TABLE I). The conidia of ''*M. anisopliae* var. *frigidum*'' were 4.5–8.0 (–9.0) × 2.0–4.0 μm and the phialides were 3.5–13.5 × 2.0–3.5 μm. The substantially larger conidia of *M. flavoviride* var. *flavoviride* was 8.0–12.0 × 3.0–6.0 μm and phialides were 7.0–19.0 × 2.0–5 μm. Conidia of the ex-type of *M. anisopliae* were 5.0–7.0 × 2.0–3.5 μm and its phialides were 8.0–11.5 × 2.0–3.0 μm. The ex-type strain of *M. flavoviride* var. *flavoviride* (ARSEF 2133) did not conidiate in culture. It has been in storage since 1956 and might have lost some of its reproductive capacity. Gams and Rozsypal (1973) expressed similar difficulties with this strain.

Phylogenetic analyses.—Sequencing of the three nuclear loci resulted in a total of 4425 unambiguously aligned characters: 711 for EF-1α introns (26 parsimony-informative characters), 990 for EF-1α (59 parsimony-informative characters), 918 for RPB1 (78 parsimony-informative characters) and 1799 for RPB2 (207 parsimony-informative characters).

In the individual analyses of the EF-1α, RPB1, and RPB2 regions (single-gene trees not shown), no conflict was found in the terminal groups (i.e. phylogenetic species). The only discrepancy was in the placement of the ''*Metarhizium anisopliae* var. *frigidum*'' clade in relation to other members of the *M. flavoviride complex*. In each analysis *M. anisopliae* was used as outgroup and *M. flavoviride* var. *pemphigi* and *M. flavoviride* var. *minus* formed sister groups. RPB1 and EF-1α placed ''*M. anisopliae* var. *frigidum*'' as sister of *M. flavoviride* var. *flavoviride*. However RPB2 placed this taxon as ancestral to *M. flavoviride* var. *pemphigi* and *M. flavoviride* var. *minus* and *M. flavoviride* var. *flavoviride* as sister of these three taxa. Because there was no conflict in the terminal groups the three genes were combined (multigene dataset).

MP analysis of the multigene dataset recovered a single most parsimonious tree of 438 steps (FIG. 9). Each taxon formed a significantly supported terminal group that received 100% support in both PP and BP. Again the only conflict found was in the placement of ''*Metarhizium anisopliae* var. *frigidum*'' in relation to the varieties of *M. flavoviride*. Bayesian analysis placed ''*M. anisopliae* var. *frigidum*'' as sister of *M. flavoviride* with 53% PP. BP provided a value of 53 in support of ''*M. anisopliae* var. *frigidum*'' placement as ancestral to *M. flavoviride* var. *pemphigi* and *M. flavoviride* var. *minus*. The *M. flavoviride* complex was significantly supported as a monophyletic grouping (100% PP and BP; see FIG. 9).

In the analysis limited to just the 5′ EF-1α intron region that included a more extensive sampling of ''*Metarhizium anisopliae* var. *frigidum*'' and *M. flavoviride* var. *flavoviride*, each taxon was supported by 99% and 100% BP, respectively (FIG. 10). In addition ''*M. anisopliae* var. *frigidum*'' strains contained a 17 base pair (bp) insertion consisting of GGGTGTCTTTTGCGTGT. Both *M. flavoviride* var. *minus* and *M. flavoviride* var. *pemphigi* included a homologous 16 bp insertion that differed only in lacking the third G (GG-TGTCTTTTGCGTGT). Strains of *M. flavoviride* var. *flavoviride* included no homologous insertion.

Based on these results we have determined that

"*Metarhizium anisopliae* var. *frigidum*" is a distinct lineage and describe it below as *Metarhizium frigidum* sp. nov.

TAXONOMY

Metarhizium frigidum J. Bisch. et S. A. Rehner, sp. nov. (FIGS. 3–6, 9)

Coloniae primum albae, deinde chlorinae-atrovirenae. Hyphae vegetativae 2.0–3.0 μm latus. Phialides ovalis-cylindricus, 3.5–13.5 μm longae et 2.0–3.5 μm crasse. Conidia longis catenis connexa columnas; subglobosus-cylindricus, 4.5–8.0(–9.0) × 2.0–4.0 μm.

Typus: AUSTRALIA. VICTORIA: BALLARAT, on an unidentified species of *Adoryphorus* (Coleoptera, Scarabaeidae), 10 Jun 1994, collected by Reinganum (BPI 872114 holotype; ARSEF 4124 ex-type).

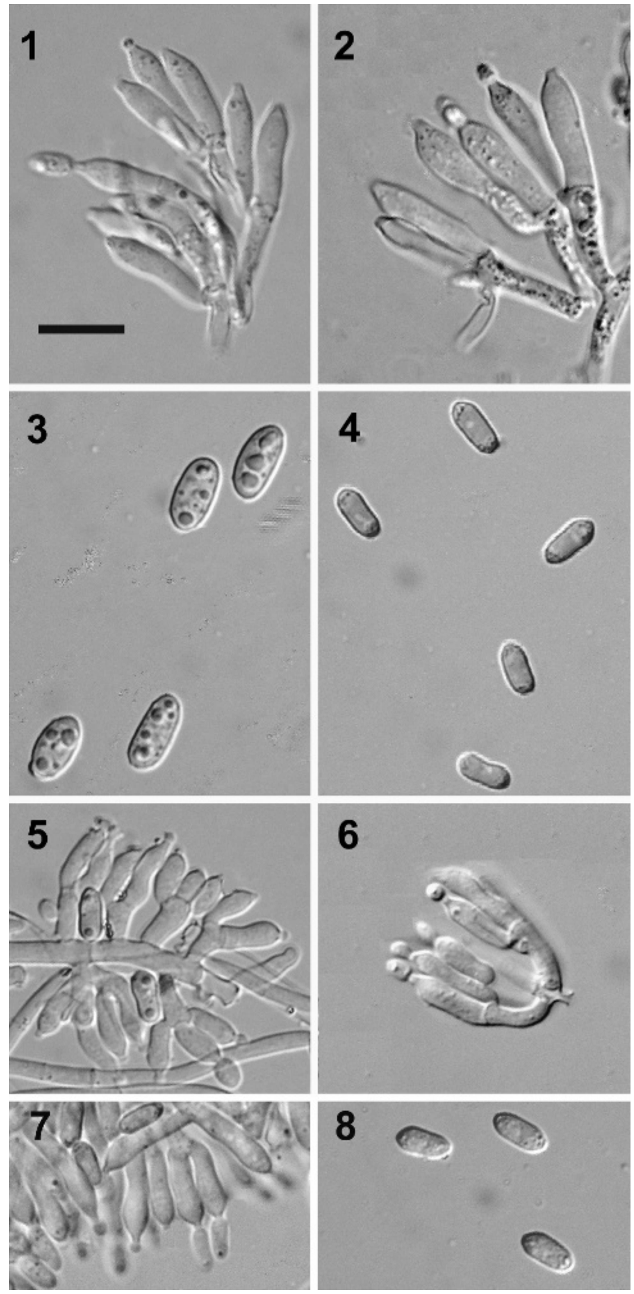
Colonies on SDY/4 medium become pigmented within 5 d to a bright green to yellow green (color plate 28E7; Kornerup and Wanscher 1967). Vegetative hyphae are smooth and 2.0–3.0 μm wide. Conidiophores bear terminal branches (1–4) each bearing 1–4 phialides, forming a palisade-like layer. Phialides are oval to cylindrical, 3.5–13.5 μm long and 2.0–3.5 μm wide. Conidia form columns in culture and are hyaline (green en masse), subglobose to cylindrical, often prominently guttulate, measure 4.5–8.0(–9.0) × 2.0–4.0 μm and many have a minute attenuating point from which the conidium was released from the phialide.

Distribution: Known only from Australia.

DISCUSSION

Based on a rDNA ITS1-5.8S-ITS2 phylogeny, Driver et al (2000) determined that the *M. anisopliae* complex is monophyletic. We used these findings as the basis for our selection of *M. anisopliae* as outgroup to clarify the relationships among *M. flavoviride* var. *flavoviride*, *M. flavoviride* var. *minus*, *M. flavoviride* var. *pemphigi* and *M. frigidum*. However the ITS regions did not provide sufficient resolution to clarify the relationships within what Driver et al (2000) called Clade 6, which contained *M. frigidum* and *M. flavoviride* var. *flavoviride*. While our dataset was unable to show the precise association of *M. frigidum* to other members of this particular complex it did identify it as a distinct lineage and placed *M. flavoviride* var. *pemphigi* and *M. flavoviride* var. *minus* (FIG. 9) as sister taxa. Based on these results it appears that ITS sequence data is useful for resolving deep nodes of the *Metarhizium* phylogeny but it is not sufficient for diagnosing some species or many of the infrageneric relationships in *Metarhizium*.

In a carbohydrate use and temperature regime study, Rath et al (1995) often referred to DAT F-001 (=ARSEF 4124) as being a representative of a group of "cold-active" *Metarhizium anisopliae* strains that they designated "*M. anisopliae* var. *frigidum*" (=*M. frigidum*). Because their study lacked a molecular phylogenetic analysis and the morphology of *M.*



FIGS. 1–8. Mature conidiogenous cells and conidia of *Metarhizium* species. Bar = 10 μm. 1–3. *Metarhizium flavoviride* var. *flavoviride*. 1–2. Mature phialides with developing conidia (ARSEF 2025 and ARSEF 2024, respectively). 3. Mature conidia (ARSEF 2025). 4–6. *M. frigidum*. 4. Mature conidia (ARSEF 4124). 5–6. Mature phialides with developing conidia (ARSEF 4124). 7–8. *M. anisopliae* var. *anisopliae*. 7. Mature phialides with developing conidia (ARSEF 7487). 8. Mature conidia (ARSEF 7487).

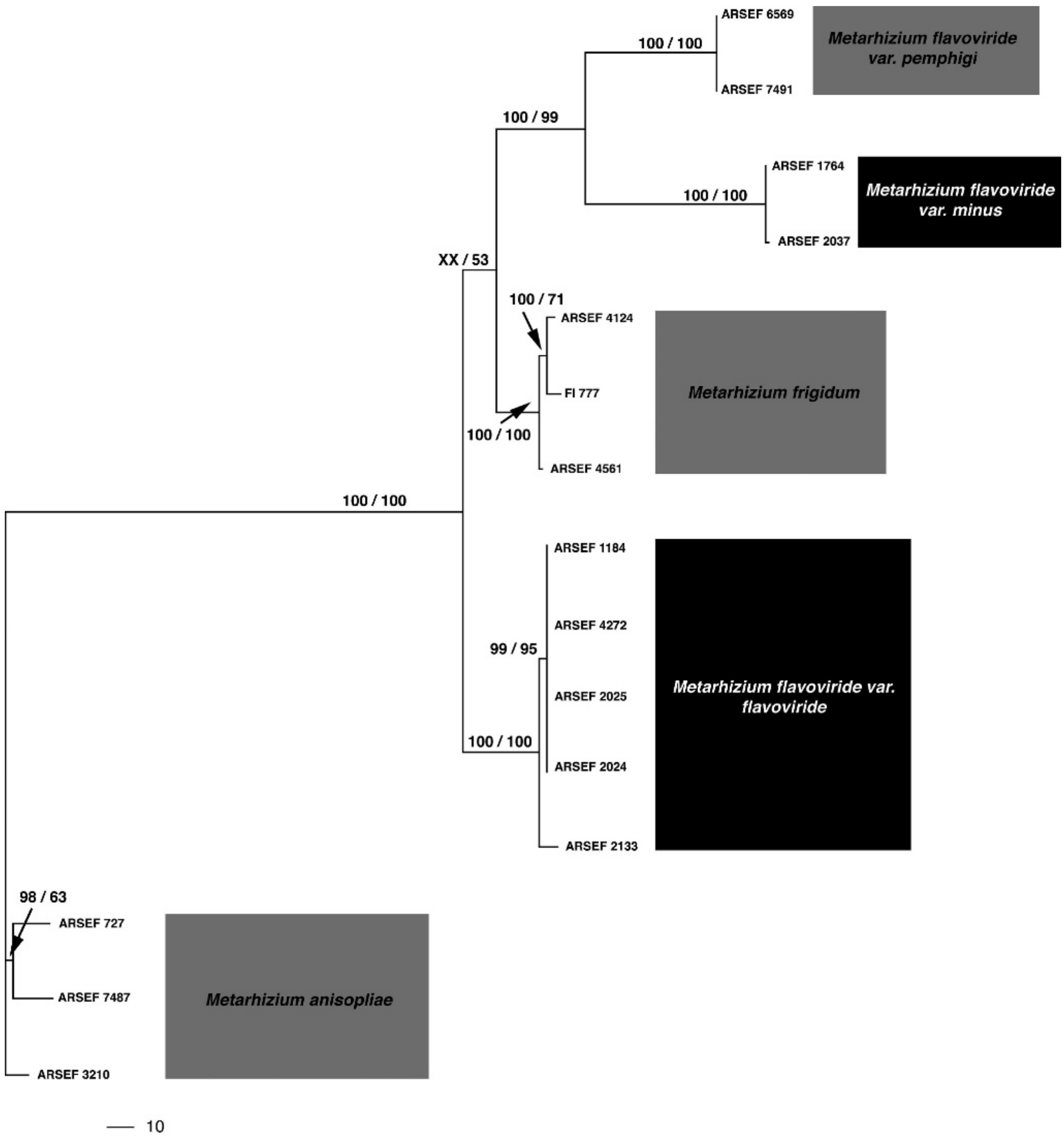


FIG. 9. Single most parsimonious tree from the combined analysis of EF-1-α, RPB1, and RPB2 (length = 438, RI = 0.942, CI = 0.893). Support values greater than 50% are shown for both PP and BP, respectively (XX = support value <50%).

frigidum is so similar to *M. anisopliae* it is easy to understand why it was considered a variety of the type species. Although this taxon was never formally described it seems likely that Rath et al (1995) would have used ARSEF 4124 as the type. Furthermore this isolate is the source for the commercially developed biological control agent BioGreen Granules™ (Rath et al 1995). For these reasons we chose to designate ARSEF 4124 to represent the type of the species.

Without molecular data the delimitation of *Metarhizium frigidum* and *M. anisopliae* is difficult. These species share similar conidia and phialide characteristics. This is likely why Rath et al (1995) associated *M. frigidum* with the *M. anisopliae* complex. However

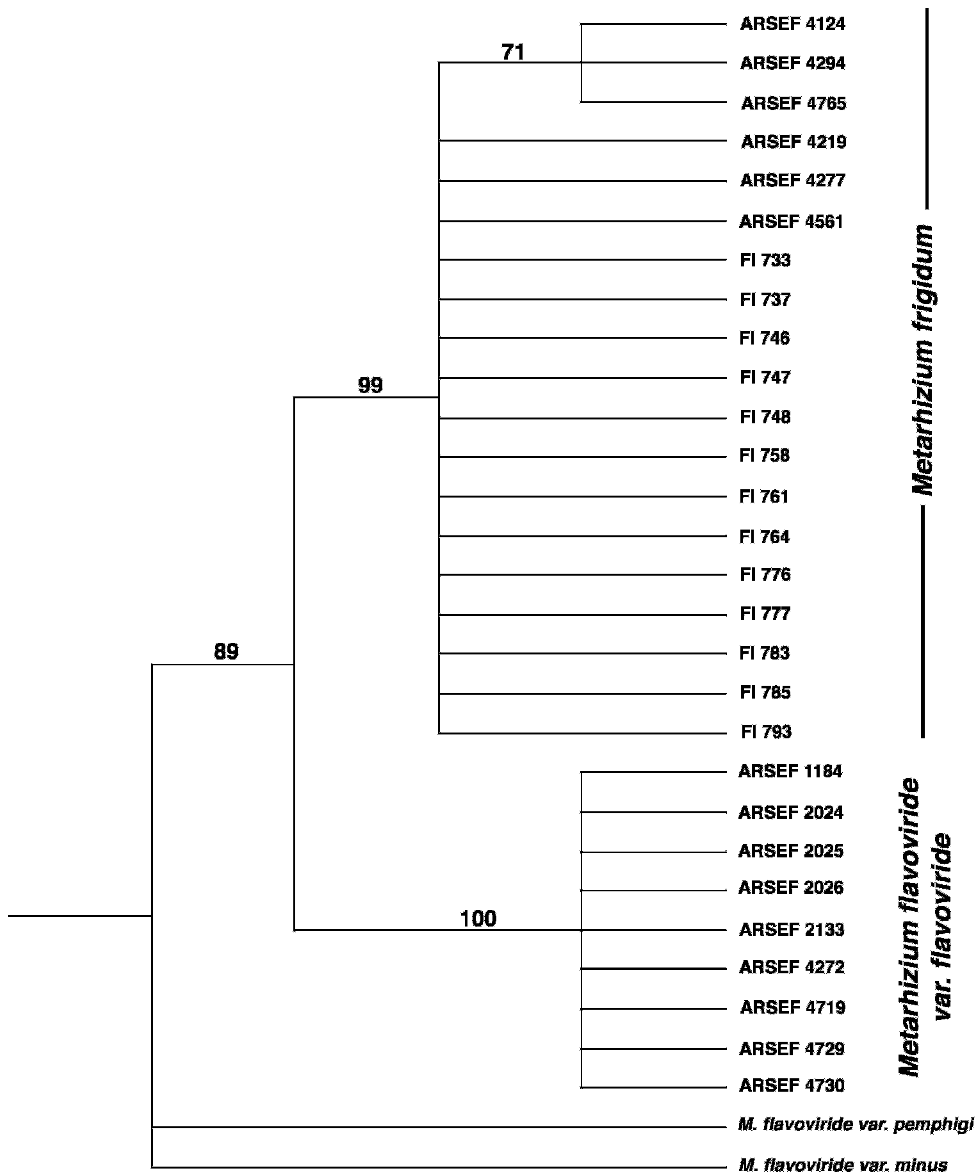
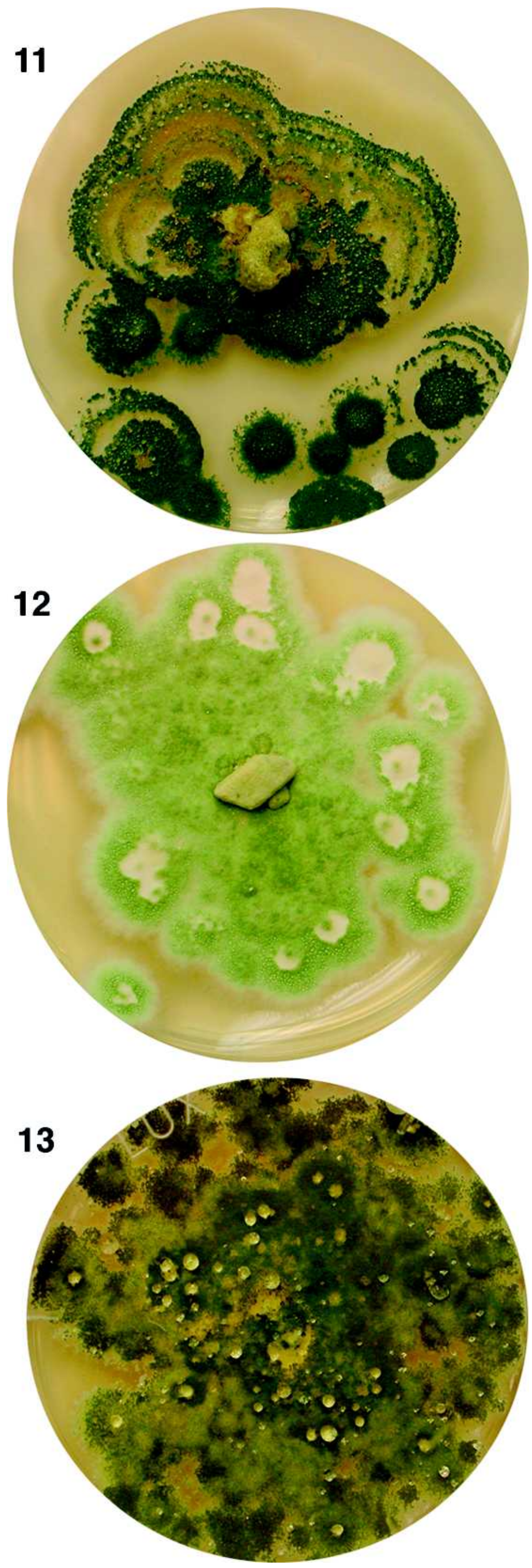


FIG. 10. Bootstrap consensus tree of the 5′ EF-1 α intron region. BP support values for the node they precede are provided. *Metarhizium flavoviride* var. *pemphigi* is represented by ARSEF 7491 and *M. flavoviride* var. *minus* is represented by ARSEF 727.

Driver et al (2000) did not distinguish *M. frigidum* from *M. flavoviride* var. *flavoviride* because the ITS sequence data did not support this distinction and they did not evaluate the morphology of these two closely related taxa. In this study it was the combined use of morphological and multilocus molecular data that helped to identify *M. frigidum* as a distinct lineage.

When comparing *Metarhizium frigidum* to *M. anisopliae* some general morphological trends become evident. The conidia and phialides of *M. anisopliae* appear to be more consistently cylindrical than those of *M. frigidum*. In addition mature cultures of *M. anisopliae* are more darkly pigmented (FIGS. 9, 11). Collection location also might be useful because *M. frigidum* appears to be restricted to Australia while *M. anisopliae* is cosmopolitan. Although their host ranges overlap *M. frigidum* has been associated only with coleopterans whereas *M. anisopliae* has a broad host range, including coleopterans. Both species can be isolated from soil. It seems clear that *M. frigidum* is a somewhat cryptic species with respect to *M. anisopliae* and culture collections might include misidentified strains. Based on these results molecular characters are the most definitive way to distinguish these taxa.

The paraphyly of the *M. flavoviride* complex as well as evaluations of the other *Metarhizium* taxa previously not included in the Driver et al (2000) study



FIGS. 11–13. Cultures of *Metarhizium* species. 11. *M. frigidum*. 12. *M. flavoviride* var. *flavoviride*. 13. *M. anisopliae* var. *anisopliae*. Isolates were grown on SDY/4 7–10 d.

will be addressed in subsequent publications (Bischoff unpubl data).

ACKNOWLEDGMENTS

The authors thank Dr Richard Milner for providing isolates. We greatly appreciate the comments and suggestions of Dr David Geiser and the anonymous reviewers. In addition we are thankful for the lab work of Ellen Buckley in helping to develop the data for this study.

The use of trade, firm or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

LITERATURE CITED

Driver F, Milner RJ, Trueman JWH. 2000. A taxonomic revision of *Metarhizium* based on a phylogenetic analysis of rDNA sequence data. Mycol Res 104:134–150.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Gams W, Rozsypal J. 1973. *Metarhizium flavoviride* n. sp. isolated from insects and from soil. Acta Botan Neerland 2:518–521.

Goettel MS, Inglis GD. 1997. Fungi: Hyphomycetes. In: Lacey L, ed. Manual of Techniques in Insect Pathology. San Diego: Academic Press. p 213–249.

Huelsenbeck JP. 2000. MrBayes: Bayesian inferences of phylogeny (software). New York: University of Rochester.

Kornerup A, Wanscher JH. 1967. Methuen Handbook of Colour. 2nd ed. London: Methuen Co.

Liang Z-Q, Liu A-Y, Liu J-L. 1991. A new species of the genus *Cordyceps* and its *Metarhizium* anamorph. Acta Sinica 10:257–262.

Liu Z-Y, Liang Z-Q, Whalley AJS, Yao Y-J, Liu A-Y. 2001. *Cordyceps brittlebankisoides*, a new pathogen of grubs and its anamorph, *Metarhizium anisopliae* var. *majus*. J Invertebrate Pathol 78:178–182.

Liu YJ, Whelen S, Hall BD. 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. Mol Biol Evol 16:1799–1808.

Mason-Gamer RJ, Kellogg EA. 1996. Testing for phylogenetic conflict among molecular data sets in the Triticeae (Gramineae). Syst Biol 45:524–545.

Metschnikoff E. 1879. Maladies des hannetons ble. Zapiski imperatorskogo obshchestua sel'ska Khozyaistra yuzhnoi rossii, 17–50.

Rath AC, Carr CJ, Graham BR. 1995. Characterization of *Metarhizium anisopliae* strains by carbohydrate utilization (AP150CH). J Invertebrate Pathol 65:152–161.

Reeb V, Lutzoni F, Roux C. 2004. Contribution of RPB2 to multilocus phylogenetic studies of the Pezizomycotina (Euascomycetes, Fungi) with special emphasis on the lichen-forming Acarosporaceae and evolution of polyspory. Mol Phylogenet Evol 32:1036–1060.

Rehner SA, Buckley E. 2005. A *Beauveria* phylogeny inferred from nuclear ITS and EF1-α sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs. Mycologia 97:84–98.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.

Sajap AS, Kaur K. 1990. Histopathology of *Metarhizium anisopliae* an entomopathogenic fungus infection in the termite *Coptotermes curvignaths*. Pertanika 13:331–334.

Stiller JWB, Hall D. 1997. The origin of red algae: implications for plastid evolution. Proc Nat Acad Sci 94:4520–4525.

Swofford DL. 2002. PAUP*: phylogenetic analysis using parsimony (*and other methods) version 4.0b10. Sunderland, Mass: Sinauer Associates Inc.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The Clustal X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acid Res 24:4876–4882.

Tulloch M. 1976. The genus *Metarhizium*. Trans Brit Mycol Soc 66:407–411.

Yip H, Rath AC, Koen TB. 1992. Characterization of *Metarhizium anisopliae* isolates from Tasmanian pasture soils and their pathogenicity to redhead cockchafer (Coleoptera: Scarabaeidae: *Adoryphorus couloni*). Mycol Res 96:92–96.